Rapid Transbilayer Movement of the Fluorescent Sterol Dehydroergosterol in Lipid Membranes

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ABSTRACT This study establishes a new assay for measuring the transbilayer movement of dehydroergosterol (DHE) in lipid membranes. The assay is based on the rapid extraction of DHE by methyl-β-cyclodextrin (M-CD) from liposomes. The concentration of DHE in the liposomal membrane was measured by using fluorescence resonance energy transfer (FRET) from DHE to dansyl-phosphatidylethanolamine, which is not extracted from liposomes by M-CD. The method was applied to small (SUV) and large (LUV) unilamellar vesicles of different compositions and at various temperatures. From the kinetics of FRET changes upon extraction of DHE from membranes, rates of M-CD mediated extraction and flip-flop of DHE could be deduced and were found to be dependent on the physical state of the lipid phase. For egg phosphocholine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine in the liquid-crystalline state, halftimes of extraction and transbilayer movement were <5 s and $\sim$20–50 s, respectively, at 10°C. For 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine-SUV being in the gel state at 10°C, the respective halftimes were 28 s and 5–8 min. Surprisingly, DHE could not be extracted from LUV consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine. This might be an indication of specific interactions between DHE molecules in membranes depending on the phospholipid composition of the membrane.

INTRODUCTION

Cholesterol constitutes a major component of mammalian plasma membranes. However, the pathways and dynamics of transport of cholesterol between different plasma membrane domains and between various intracellular compartments in those cells are not known. In particular, the kinetics and mechanism of transbilayer movement of cholesterol, which is an essential step in intracellular trafficking, is still controversial. Measured halftimes of flip-flop differ by at least one order of magnitude. Studies on lipid vesicles reported halftimes of several seconds to a few minutes (Backer and Dawidowicz, 1979; Kan et al., 1992) or halftimes in the order of several hours (Poznansky and Lange, 1976, 1978; Rodrigueza et al., 1995). As for model membranes, experimental data on cholesterol flip-flop in red blood cells are inconsistent with halftimes varying from seconds to several hours (Lange et al., 1981; Brasaemle et al., 1988; Schroeder et al., 1991; Yancey et al., 1996). It is not entirely clear what reasons account for these contradicting data. However, recent work indicates that flip-flop of cholesterol is rapid with halftimes of the order of a few minutes or even seconds (Leventis and Silvius, 2001). In the light of these investigations there is still a need for appropriate assays allowing the determination of a rapid cholesterol flip-flop.

In recent years dehydroergosterol (DHE) has become a popular cholesterol analog. DHE is a naturally occurring sterol composing up to 20% of the total sterol in yeast. It is fluorescent due to a conjugated triene system that leaves the 3-β-hydroxyl group and the alkyl tail of the cholesterol backbone unperturbed. DHE differs from cholesterol only in having three additional double bonds and an extra methyl group. Unlike other fluorescent cholesterol analogs, it does not have a bulky reporter group and shows a similar physicochemical behavior similar to cholesterol with respect to its lateral and transverse organization in model membranes (Hale and Schroeder, 1982; Loura and Pietro, 1997; Cheng et al., 1999). It can be incorporated into plasma membranes of LM fibroblasts in large quantities without altering cell growth, cell doubling time, and change in activity of plasma membrane proteins (Hale and Schroeder, 1982), and its distribution in CHO cells was recently studied by fluorescence microscopy (Mukherjee et al., 1998). Moreover, DHE can replace native sterols of cultured cells without perturbing their growth activities and can be utilized by *Caenorhabditis elegans* as the only sterol source (Matyash et al., 2001).

The purpose of this study was to establish a new assay for monitoring the transbilayer movement of DHE in lipid membranes of various compositions online. Therefore, we measured the extraction of DHE from liposomes by methyl-β-cyclodextrin (M-CD). β-Cyclodextrins have been used for the efficient and rapid modulation of the cholesterol content in membranes (Kilsdonk et al., 1995; Ohvo and Slotte, 1996). Maximum rates for cyclodextrin-mediated efflux are 3.5- to 70-fold higher than for efflux induced by HDL_3 as found for different cell lines (Kilsdonk et al.,

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1995). Among three types of β-cyclodextrins (β-cyclodextrin, M-CD, and 2-hydroxypropyl-β-cyclodextrin) M-CD was most efficient in extracting cellular cholesterol. In this study we monitored the extraction of DHE from membranes by using a fluorescence resonance energy transfer (FRET) between DHE and the fluorescent phospholipid analog *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-*sn*-glycero-3-phosphoethanolamine (dansyl-PE). Different from DHE, dansyl-PE is not extracted from membranes by M-CD. Thus, upon extraction of DHE from membranes, FRET between both probes is decreasing. With the help of an appropriate kinetic model the extraction kinetics observed by FRET were used to calculate transbilayer migration rates of DHE.

MATERIALS AND METHODS

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), egg phosphocholine (eggPC), dehydroergosterol (DHE), and methyl-β-cyclodextrin (M-CD) were obtained from Sigma Chemicals (Deisenhofen, Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(2,4,6-trinitrophenyl) (TNP-PE) was purchased from Avanti Polar Lipids (Alabaster, AL). *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-*sn*-glycero-3-phosphoethanolamine (dansyl-PE) was purchased from Molecular Probes (Leiden, The Netherlands) and 6-carboxyfluorescein (6-CF) was purchased from Calbiochem (Schwalbach, Germany). All lipids were dissolved in chloroform and stored at −20°C. PBS contained 5.8 mM Na_2HPO_4/NaH_2PO_4 and 150 mM NaCl and was set to pH 7.4. M-CD was dissolved in aqua dest. yielding a stock solution of 350 mM.

Composition of liposomes

For measuring extraction of DHE, liposomes contained DHE, dansyl-PE, and either DMPC, eggPC, or POPC. Dansyl-PE and DHE composed 2.5 mol % and up to 9 mol %, respectively, of the total lipid. For quenching of DHE or dansyl-PE, liposomes were prepared that contained either dansyl-PE or DHE and TNP-PE and eggPC. For quenching of dansyl-PE fluorescence, liposomes contained 2.7 mol % dansyl-PE and 10 mol % TNP-PE, whereas for quenching of the DHE fluorescence 17 mol % TNP-PE and 9 mol % DHE of the total lipid were used. For the leakage assay, liposomes of POPC, DMPC, or eggPC and 9 mol % DHE of the total lipid were prepared.

Preparation of small unilamellar vesicles (SUV)

Lipid mixtures of the desired composition were dried under nitrogen. To prevent the lipids from sticking to the bottom of the glass tube they were dissolved in a small volume of absolute ethanol, then resuspended in PBS and vortexed for ~30 s, yielding a maximum final ethanol concentration of 1%. Lipid suspensions were sonicated (Branson Sonifier 250, Schwäbisch Gmünd, Germany, intensity 2, cycle 80%) on ice for 20 min. SUV were stored on ice and used within 4 h of preparation.

Preparation of large unilamellar vesicles (LUV)

Lipid suspensions of the desired composition in PBS were prepared as described for SUV, but before the preparation the buffer was filtered and degassed. After addition of PBS, the lipid dispersion was vortexed for ~60 s and then went through five freeze-thaw cycles. A freeze-thaw cycle included an incubation period at −70°C for 10 min, followed by an incubation period at 50°C for 5 min. Subsequently, the lipid suspension was extruded (Extruder from Lipex Biomembranes Inc., Vancouver, Canada) 10 times through polycarbonate filters (Nucleopore GmbH, Tübingen) with a pore size of 100 nm at 50°C. LUV were stored at 6°C and used within two weeks of preparation.

Leakage assay

SUV were prepared in PBS containing additionally 6-CF at a self-quenching concentration (10 mM). SUV were separated from non-encapsulated 6-CF by column chromatography on a PD-10 column (Sephadex) at room temperature using PBS as elution buffer and a column chromatography facility of BioRad (München, Germany). Elution of SUV was monitored by a UV detector. Leakage of the liposomal content was measured by mixing SUV and M-CD in a cuvette and following the increase in 6-CF fluorescence due to the dilution of the fluorophore and accompanied decrease of quenching. Complete leakage was induced by addition of Triton X-100 (final concentration 1%). The degree of leakage (L) was estimated using Eq. (1):

$$L = \frac{f_1 - f_0}{f_{\max} - f_0} 100\% \tag{1}$$

where f_0 and f_1 denote the fluorescence intensity before and after M-CD addition, and $f_{\max}$ denotes the fluorescence after addition of Triton X-100.

Fluorescence measurements

Fluorescence was measured using an Aminco-Bowman Series 2 spectrometer (SLM-AMINCO, Rochester, NY) with a stirring unit and a continuous-wave 150 W xenon lamp as a light source. The detector voltage was set to 750 V for all experiments. For the leakage assay, slit sizes were 1 nm and 2 nm for excitation and emission, respectively. For all other experiments, slit sizes were 4 nm for both excitation and emission; 6-CF fluorescence was determined by using an excitation and emission wavelength of 492 nm and 516 nm, respectively. The total absorbance at the excitation wavelength was kept as low as possible to avoid artifacts due to inner filter effects. To measure the energy transfer from DHE to the dansyl moiety, dual excitation time scans with a resolution of 1 s or 0.5 s were performed. The excitation wavelengths were 332 nm and 344 nm, respectively. The emission wavelength was set to 498 nm. Measurements were performed at 10, 23, and 30°C. The maximum lipid concentration was 75 μM. For details of the extraction assay using M-CD, see Results.

Model equations for data-fitting

To estimate rate constants of DHE extraction and transbilayer movement, and the DHE distribution in the membrane, a simple kinetic model was used (Marx et al., 2000). The model considers the transbilayer movement of DHE from the inner to the outer leaflet of the membrane, and vice versa, by the rate constants k_{+1} and k_{-1}, respectively; c_i and c_o are the DHE concentrations in the inner and outer leaflet of the liposome membrane, respectively; c denotes the total concentration of DHE and is a conserved quantity. The DHE molecules located on the outer leaflet are solely available for extraction by M-CD. This extraction is assumed to be pseudo-monomolecular and reversible with the rate constants k_{+2} and k_{-2}, respec-

tively. The model is represented by an inhomogeneous system of ordinary differential first-order equations with constant coefficients (Eq. 2).

$$\begin{pmatrix} \frac{dc_i}{dt} \\ \frac{dc_o}{dt} \end{pmatrix} = \begin{bmatrix} -k_{+1} & k_{-1} \\ k_{+1} - k_{-2} & -(k_{-1} + k_{+2} + k_{-2}) \end{bmatrix} \cdot \begin{pmatrix} c_i \\ c_o \end{pmatrix} + \begin{pmatrix} 0 \\ c \cdot k_{-2} \end{pmatrix} \quad (2)$$

Before extraction ($k_{+2} = k_{-2} = 0$) the system is assumed to be in steady state, which determines the initial conditions:

$$c_i(t = 0) = p_i = c \frac{k_{-1}}{k_{+1} + k_{-1}}$$

and (3)

$$c_0(t = 0) = p_o = c \frac{k_{+1}}{k_{+1} + k_{-1}}$$

The proportion of DHE bound to M-CD at equilibrium (c_m) can be estimated using the following equation:

$$c_m = \frac{k_{+2}}{k_{-2}} \cdot \frac{c}{1 + k_{-1}/k_{+1} + k_{+2}/k_{-2}} \quad (4)$$

The corresponding halftimes $T_{0.5}$ for the respective rate constants k of reactions are determined by:

$$T_{0.5} = \frac{\ln 2}{k} \quad (5)$$

Systems of the form $y' = Ay + e(t)$ with the initial conditions $y(t = 0) = y_0$ can be solved by the "Method of Impulses," yielding the solution:

$$c_i = X \cdot e^{\lambda_1 t} + Y \cdot e^{\lambda_2 t} + \frac{ck_{-2}k_{+1}}{\lambda_1\lambda_2}$$

and (6)

$$c_o = f_1 \cdot X \cdot e^{\lambda_1 t} + f_2 \cdot Y \cdot e^{\lambda_2 t} + \frac{ck_{-2}k_{+1}}{\lambda_1\lambda_2}$$

with

$$X = p_o - f_2 p_i + c \frac{k_{-2}}{\lambda_1(f_1 - f_2)}$$

and (7)

$$Y = f_1 p_i - p_o - c \frac{k_{-2}}{\lambda_2(f_1 - f_2)}$$

The function $f = c_i + c_o$ was fitted to the experimental extraction kinetics by using the rate constants k_{+1}, k_{-1}, k_{+2} and k_{-2} as fit parameters. All regressions were performed using SigmaPlot (Jandel Scientific, Erkrath, Germany) and a Marquardt-Levenberg algorithm.

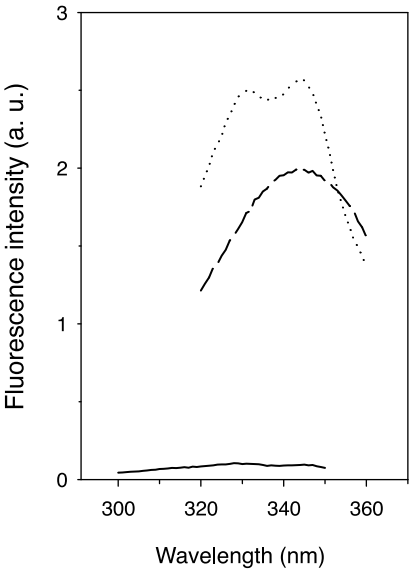

FIGURE 1 Excitation spectra of dansyl-PE (*dashed line*), DHE (*solid line*), and a mixture of DHE and dansyl-PE (*dotted line*) in eggPC-SUV at 10°C (emission at 498 nm). Phospholipid was 75 μM, dansyl-PE 2.5 mol %, and DHE 9 mol %.

RESULTS

Extraction assay

DHE displays the same spectral properties when incorporated into membranes and M-CD (spectra not shown). Therefore, the extraction of DHE from membranes by cyclodextrins cannot be followed simply by monitoring the DHE fluorescence. However, fluorescence energy transfer offers the possibility to monitor and quantify extraction of DHE from membranes if the respective FRET partner molecule is localized in the membrane before and after addition of M-CD. It is known that DHE and the dansyl group can mediate Foerster energy transfer, with DHE serving as donor and dansyl serving as acceptor molecule (Wrenn et al., 1999). Both fluorophores are excitable between 300 and 360 nm. The DHE excitation spectrum has two peaks at 330 and 344 nm, respectively, and one shoulder at 315 nm, whereas dansyl-PE has one peak at 344 nm (see Fig. 1). In the emission spectrum DHE reveals three peaks between 350 and 400 nm, but is nonfluorescent at 500 nm, whereas dansyl displays an emission peak around 500 nm (see Fig. 2). The small overlap between the emission spectrum of DHE and the excitation spectrum of dansyl forms the basis for an energy transfer. In liposomes containing dansyl-PE and DHE this energy transfer is evident in the excitation spectrum of dansyl-PE, which displays two peaks at 332 nm and 344 nm instead of just one peak at 344 nm (Fig. 1). The shape of the dansyl excitation spectrum is sensitive to the transfer efficiency and reflects properties of the DHE excitation spectrum.

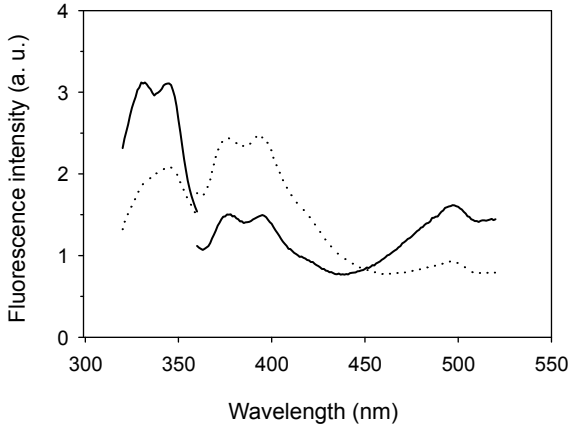

FIGURE 2 Excitation spectra (emission at 498 nm) and emission spectra (excitation at 310 nm) of eggPC-LUV containing DHE and dansyl-PE before (*solid line*) and after (*dotted line*) the addition of M-CD at 10°C. Phospholipid was 75 μM, dansyl-PE 2.5 mol %, DHE 9 mol %, and M-CD 20 mM.

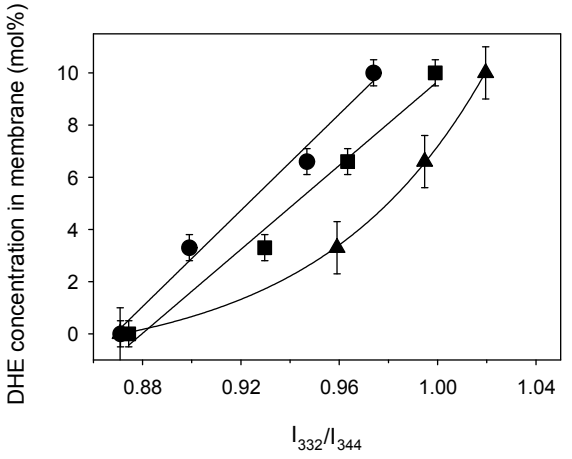

FIGURE 3 Relation between DHE concentration and the ratio $r = I_{332}/I_{344}$ of the dansyl excitation spectrum in eggPC- (*circles*), DMPC- (*squares*), and POPC-SUV (*triangles*) at 10°C. Emission was 498 nm, the phospholipid concentration 75 μM. The lines represent fits of the data to a linear function (eggPC, DMPC) and to an exponential function (Eq. 9, POPC). Results of the regression: EggPC $n = -80 \pm 7$ mol %, $m = 92 \pm 8$ mol %, CR = 0.99; DMPC $n = -71 \pm 7$ mol %, $m = 80 \pm 7$ mol %, CR = 0.99; POPC $y_0 = -1.3 \pm 0.2$ mol %, $a = (3 \pm 2)10^{-6}$ mol %, $b = 14.9 \pm 0.5$, CR = 1), with parameters m and n being the slope and the axis intercept, respectively, and parameters y_0, a, and b as introduced in Eq. 9; CR denotes the correlation coefficient. Error bars are based on an estimation of a maximum error due to pipetting small label volumes. Note: for determination of DHE concentration in membranes from measured intensity ratio r, the DHE concentration is presented as a function of the intensity ratio r.

The addition of M-CD to liposomes containing phospholipid, dansyl-PE, and DHE remarkably changed the combined spectra of DHE and dansyl-PE (Fig. 2). First, the intensity of the dansyl-PE excitation spectrum was decreasing and the peak at 332 nm was vanishing. Second, the emission intensity of DHE was increasing with a concomitant decrease in the fluorescence intensity of dansyl-PE. The changes in fluorescence intensity and the changes in the shape of the spectra are indicators for a decreased fluorescence energy transfer between DHE and dansyl-PE. Upon extraction of DHE from the liposomes the close distance between donor and acceptor molecules is lost, resulting in a decrease of the transfer efficiency. The addition of M-CD to liposomes containing only phospholipid and dansyl-PE did not significantly alter the excitation and emission spectra of this fluorophore (spectra not shown). An energy transfer is also obvious from an increase in fluorescence emission of dansyl-PE at 498 nm when excited at 310 nm in liposomes containing dansyl-PE and DHE compared with liposomes containing only dansyl-PE after extraction of DHE by M-CD.

The ratio of the fluorescence intensities at 332 nm and 344 nm ($r = I_{332}/I_{344}$) of the dansyl-PE excitation spectrum (measured at an emission of 498 nm) is sensitive to the DHE content in the liposomal membrane. Fig. 3 depicts the ratio for different DHE concentrations in the membrane for SUV consisting of eggPC, DMPC, or POPC at 10°C. As shown, the relationship between the DHE content in the membrane and the intensity ratio can be described as a linear or an exponential function, depending on the lipid composition of the vesicles. A linear description is adequate for eggPC and DMPC. With this linear relationship changes of the DHE concentration ($c(t)$) upon addition of M-CD can be calculated from the intensity ratio ($r(t)$) according to Eq. 8:

$$c(t) = \frac{r(t) - r_0}{r_{\max} - r_0} \tag{8}$$

The intensity ratio (I_{332}/I_{344}) estimated from the dansyl-PE spectrum in liposomes in the absence of DHE (corresponding to complete extraction of DHE from membranes) yields the reference state r_0, and the intensity ratio before the extraction denotes $r_{\max}$.

Unfortunately, a linear description does not seem to be appropriate for POPC liposomes. A more accurate description for the relationship DHE concentration versus intensity ratio is an exponential function of the form:

$$c = y_0 + a \cdot e^{br} \tag{9}$$

As in the case for a linear fit, the DHE concentration ($c(t)$) can be determined from $r(t)$ by a simple equation (10), but two parameters are necessary: r_0, the intensity ratio of the pure dansyl-PE spectrum (see above) and b, the exponential factor as introduced in Eq. 9.

$$c(t) = \frac{e^{b(r(t)-r_0)} - 1}{e^{b(r_{\max}-r_0)} - 1} \tag{10}$$

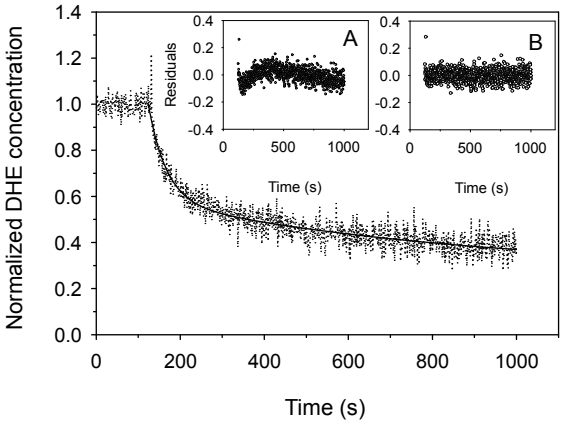

FIGURE 4 Extraction of DHE from DMPC-SUV by M-CD at 10°C. 75 μM DMPC-SUV containing 9 mol % DHE and 2.5 mol % dansyl-PE were mixed with 24 mM M-CD, and the time-dependent decrease of the DHE concentration in the vesicle membrane was determined as described in the text. The line represents the fit of experimental data according to the model described in Materials and Methods. *Insets A* and *B* show residuals of a single-exponential fit and of the fit according to the model, respectively.

Although the spectral properties of dansyl-PE in membranes do not change significantly upon addition of M-CD, the intensity ratio r_0 is subject to minor changes (data not shown). For both SUV and LUV, r_0 is decreasing with increasing M-CD concentrations. For example, without M-CD r_0 is ~0.8706 and with 24 mM M-CD (the highest concentration used for extraction) r_0 is ~0.8595. For eggPC-LUV containing 9 mol % DHE, r_{max} was determined to be 0.9926 ± 0.0007. If this dependence of r_0 on M-CD is not taken into account, the maximum deviation is ≤10% because

$$1.09 \approx \frac{0.9926 - 0.8595}{0.9926 - 0.8706}$$

To minimize this deviation, r_0 measured in the presence of 19 mM M-CD was used for correcting the calibration curves in Fig. 3 as well as for calculating the decrease of the DHE concentration in membranes due to extraction of DHE by M-CD (see below).

Using this approach, the extraction of DHE from liposomes can be followed online by performing a dual excitation time scan with the wavelengths set to 332 nm and 344 nm, and with an emission wavelength of 498 nm (see Fig. 4). r_{max} was determined by averaging r over the first 100 s. Subsequently, M-CD was added to the suspension and the intensity ratio ($r(t)$) was followed. By use of Eqs. 8 or 10, respectively, the intensity ratio can be converted into the amount of DHE in the membrane at time t. The DHE concentration in the membrane before the extraction $c(t = 0)$ was normalized to 1. Fig. 4 shows an extraction kinetics for DHE from DMPC-SUV, exemplary, at 10°C. Upon addition of M-CD, an initial rapid decrease followed by a second slower decline of the DHE concentration in the SUV membrane was observed. The initial phase is related to the rapid extraction of DHE from the outer leaflet, whereas the second phase reflects the transbilayer movement of DHE. Indeed, this kinetics can be fitted by a biexponential function, which is the analytical solution of a simple three-compartment model (see Materials and Methods). The quality of the data fit is visualized in *inset B* of Fig. 4. For comparison, *inset A* of Fig. 4 displays the residuals for a single-exponential fit. A random distribution of the residuals was found only for the biexponential fit, not for the single-exponential fit.

Dansyl-PE is not extracted by M-CD

For the extraction assay described it is vital that dansyl-PE is not extracted by M-CD from liposomes. To verify this, extraction experiments were performed with eggPC-SUV containing DHE or dansyl-PE and TNP-PE as fluorescence quencher. TNP-PE quenched both fluorophores in membranes. However, a rather high TNP-PE concentration of 17 mol % had to be used for efficient quenching. As was anticipated, the addition of M-CD to SUV containing dansyl-PE and TNP-PE did not cause a significant increase in fluorescence due to dequenching, whereas the addition of M-CD to SUV containing DHE and TNP-PE led to a rapid dequenching of the DHE fluorescence (data not shown). These results demonstrate that dansyl-PE is not extracted by M-CD. However, dequenching of DHE was faster in comparison to the decrease of FRET between DHE and dansyl-PE upon extraction by M-CD, thus preventing the determination of the transbilayer movement of DHE. We surmise that the rather high TNP-PE concentration led to a perturbation of the bilayer structure, which affected the extraction and transbilayer movement of DHE.

Leakage of vesicles in the presence of M-CD

For measuring DHE flip-flop rates with the approach described, it is essential that the liposomes remain intact in the presence of M-CD. Any destruction of liposomes would interfere with the extraction kinetics and would have an impact on the determination of the respective rate constants. Therefore, leakage of 6-CF from SUV consisting of DMPC, eggPC, or POPC each containing 9 mol % DHE was measured. Leakage was dependent on the temperature and the M-CD concentration. Fig. 5 depicts the percent lysis of eggPC liposomes for different M-CD/lipid ratios and temperatures. At 10°C, within the time scale of the DHE extraction, leakage did not exceed 8% at M-CD concentrations used for extraction experiments (highest M-CD/lipid-ratio was ~500, see (+) in Fig. 5) as found for DMPC-, eggPC-, and POPC-SUV (data only shown for eggPC-SUV in Fig.

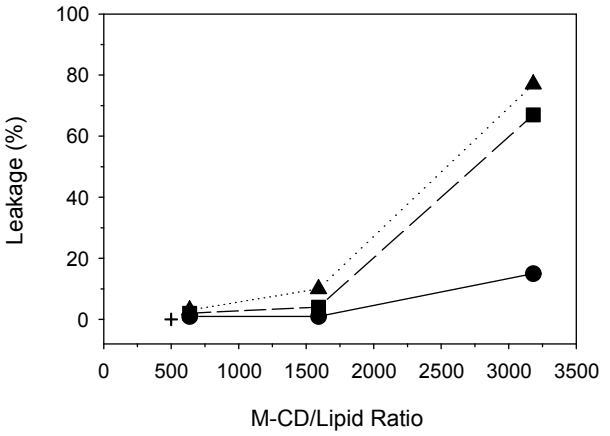

FIGURE 5 Leakage of 6-CF from eggPC-SUV at varying M-CD/lipid ratios at 10°C (*circles*), 23°C (*squares*), and 30°C (*triangles*). SUV loaded with 6-CF were prepared and the leakage of the fluorophore as measured by the increase of fluorescence at different concentrations of M-CD was performed as described in Materials and Methods. Complete leakage (100%) was induced by addition of Triton X-100 (final concentration 1%). (+) indicates the highest M-CD/lipid ratio used for extraction experiments.

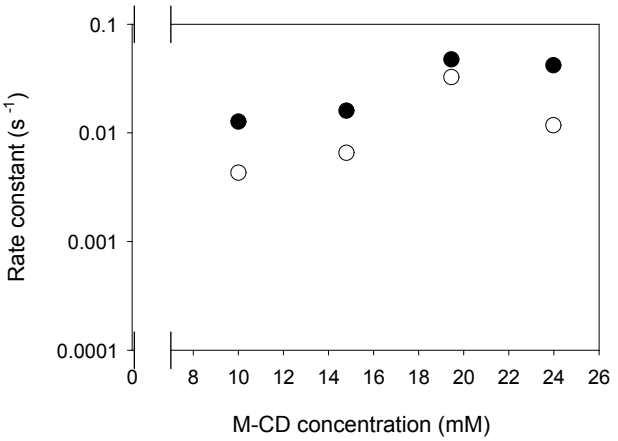

FIGURE 6 Rate constant of flip (k_{-1}, *open circles*) and flop (k_{+1}, *filled circles*) of DHE in eggPC-SUV at different M-CD concentrations at 10°C. Seventy-five μM eggPC-SUV containing 9 mol % DHE and 2.5 mol % dansyl-PE were mixed with M-CD, and the time-dependent decrease of the DHE concentration in the vesicle membrane was determined as described in the text. The rate constants for flip and flop were determined from fitting of extraction kinetics (see Fig. 4) according to the model described in Materials and Methods.

5). Therefore, vesicle leakage did not have a major impact on extraction kinetics, even at higher temperatures.

However, lysis became significant with increasing temperature and increasing M-CD/lipid-ratios. Above an M-CD/lipid ratio of 1600 a dramatic increase of the leakage was observed at 23°C and 30°C (only shown for eggPC, Fig. 5).

Flip-flop of DHE in SUV membranes

To measure the flip-flop of DHE in SUV consisting of DMPC, eggPC, or POPC, the DHE extraction assay using FRET (as described above) was performed at 10°C. SUV contained 9 mol % DHE and 2.5 mol % dansyl-PE. Lipids were chosen with respect to their phase behavior. At 10°C, DMPC is in the gel state, whereas POPC is in the liquid-crystalline state. EggPC consisting of PC molecules with varying acyl chain lengths and degrees of saturation does not display a distinct thermal phase behavior with a gel and liquid-crystalline phase, but is assumed to exist in a liquid-crystalline state. For these three lipid species extraction kinetics with varying M-CD concentrations were recorded and analyzed using the model equations as described above. Fig. 6 depicts the rate constants for flip and flop of DHE between the inner and outer leaflet exemplary in eggPC-

TABLE 1 Flip-flop of DHE in SUV at 10°C measured by extraction of DHE to M-CD

Lipid	k_{+1} (10^{-3} s^{-1})	$T_{0.5}$ (s)	k_{-1} (10^{-3} s^{-1})	$T_{0.5}$ (s)	N
EggPC	29.6 ± 8.9	23.4 ± 7.0	13.8 ± 6.5	50.2 ± 23.6	4
POPC	24.7 ± 9.5	28.1 ± 10.8	2.9 ± 1.2	238.4 ± 98.8	7
DMPC	1.6 ± 0.4	423.1 ± 101.7	0.6 ± 0.1	1071.4 ± 235.8	5

k_{+1} and k_{-1} denote the rate constants for flop and flip, respectively, with $T_{0.5}$ denoting the respective halftimes. Values of parameters were obtained by fitting kinetics as shown exemplary in Fig. 4 to the model described in Materials and Methods. The mean ± SE of estimates is given. N refers to the number of experiments.

TABLE 2 Extraction of DHE from SUV at 10°C

Lipid	k_{+2} (10^{-2} s^{-1})	$T_{0.5}$ (s)	k_{-2} (10^{-4} s^{-1})	$T_{0.5}$ (s)	c_m	N
EggPC	41.2 ± 17.3	1.7 ± 0.7	18.5 ± 3.8	374.7 ± 76.2	0.99	4
POPC	25.8 ± 8.6	2.7 ± 0.9	5.4 ± 2.2	1290.7 ± 530.0	0.99	7
DMPC	2.5 ± 0.3	27.9 ± 2.9	4.8 ± 2.0	1456.5 ± 626.0	0.97	5

k_{+2} and k_{-2} denote the rate constants for extraction and reincorporation, respectively, with $T_{0.5}$ denoting the respective halftimes. c_m is the portion of DHE bound to M-CD after the extraction process is completed (at equilibrium). Values of parameters were obtained by fitting kinetics as shown exemplary in Fig. 4 to the model described in Materials and Methods. The mean ± SE of estimate is given. N refers to the number of experiments.

TABLE 3 Flip-flop of DHE in eggPC-SUV at 10 and 23°C measured by extraction of DHE to M-CD

Temperature (°C)	k_{+1} (10^{-3} s^{-1})	$T_{0.5}$ (s)	k_{-1} (10^{-3} s^{-1})	$T_{0.5}$ (s)	N
10	29.6 ± 8.9	23.4 ± 7.0	13.8 ± 6.5	50.2 ± 23.6	4
23	34.5 ± 11.6	20.1 ± 6.8	4.7 ± 3.7	147.8 ± 117.0	8

For the description of parameters see legend to Table 1.

SUV at various M-CD concentrations. Only a weak dependence of the rate constants of flip and flop on the M-CD concentration was found.

Likewise, rate constants of extraction and reincorporation of DHE, k_{+2} and k_{-2}, respectively, did not show a strong dependence on the M-CD concentration used for extraction (data not shown). This suggests that in the concentration range used, the M-CD concentration is not a rate-limiting determinant of DHE extraction. We have summarized the respective values for rate constants and halftimes of flip-flop and of extraction in Tables 1 and 2, respectively. For both eggPC- and POPC-SUV, the flop halftimes of DHE were very short: 23 and 28 s, respectively, at 10°C. For DMPC-SUV, DHE had a flop halftime of ~400 s. This strongly suggests that the rate of transbilayer movement of DHE depends on the phase state of the lipid bilayer.

From Table 2 it is evident that the halftime of extraction of DHE from membranes by M-CD is more than one magnitude smaller in comparison to the respective halftimes of flip-flop, which is an essential prerequisite for measuring flip-flop by this assay (see also Marx et al., 2000). Using Eq. 4, one can easily estimate the portion c_m of DHE bound to M-CD after the extraction process is completed (at equilibrium). Table 2 shows that almost all of the DHE was extracted from SUV for all three lipid species.

Because SUV are very small, with a diameter of ~250 Å, one would expect a ratio of the mean rate constants of flop and flip k_{+1}/k_{-1} of ~2. For eggPC-SUV and DMPC-SUV this ratio was 2.1 and 2.7, respectively, corresponding to ~70% of the DHE being localized on the outer leaflet. For POPC-SUV this ratio was 8.3, which could imply that ~90% of the DHE was localized on the outer leaflet. Because we expected a homogeneous distribution of DHE between both leaflets this high ratio indicated that the extraction is too rapid and flip rates could not be measured reliably.

Influence of temperature on the DHE flip-flop in eggPC-SUV

To estimate the influence of temperature on the DHE flip-flop the extraction assay was performed for eggPC-SUV at 10 and 23°C. Tables 3 and 4 depict the results of the extraction kinetics. The extraction of DHE was similar at 23°C in comparison to 10°C (see Table 4). Likewise, the flop rate constants were virtually the same for both temperatures, whereas the rate constant for the flip, i.e., the movement from the outer to the inner monolayer, was smaller at 23°C. The associated ratio k_{+1}/k_{-1} was 7.3 at 23°C, again indicating that the extraction was too rapid and flip rates could not be measured reliably at this temperature.

TABLE 4 Extraction of DHE from eggPC-SUV at 10 and 23°C

Temperature (°C)	k_{+2} (10^{-2} s^{-1})	$T_{0.5}$ (s)	k_{-2} (10^{-4} s^{-1})	$T_{0.5}$ (s)	c_m	N
10	41.2 ± 17.3	1.7 ± 0.7	18.5 ± 3.8	374.7 ± 76.2	0.99	4
23	47.9 ± 11.1	1.5 ± 0.3	43.1 ± 18.2	160.8 ± 67.9	0.99	8

For the description of parameters see legend to Table 2.

TABLE 5 Flip-flop of DHE in LUV at 10°C measured by extraction of DHE to M-CD

Lipid	k_{+1} (10^{-2} s^{-1})	$T_{0.5}$ (s)	k_{-1} (10^{-3} s^{-1})	$T_{0.5}$ (s)	N
POPC	2.0 ± 0.6	34.1 ± 10.2	17.9 ± 4.9	38.7 ± 10.6	7
EggPC	1.4 ± 0.7	50.2 ± 24.3	10.6 ± 5.1	65.4 ± 31.5	7

For the description of parameters see legend to Table 1.

TABLE 6 Extraction of DHE from LUV at 10°C

Lipid	k_{+2} (10^{-2} s^{-1})	$T_{0.5}$ (s)	k_{-2} (10^{-3} s^{-1})	$T_{0.5}$ (s)	c_m	N
POPC	17.3 ± 1.9	4.0 ± 0.4	3.0 ± 0.5	228.2 ± 39.2	0.97	7
EggPC	19.8 ± 14.1	3.5 ± 2.5	3.8 ± 1.6	183.0 ± 78.7	0.93	7

For the description of parameters see legend to Table 2.

Flip-flop of DHE in LUV membranes

The extraction experiment for SUV was also performed for LUV at 10°C. The results for the extraction of DHE from POPC- and eggPC-LUV are displayed in Tables 5 and 6. For both eggPC- and POPC-LUV flip-flop rates were of the same order of magnitude as found for the respective SUV, and were below 1 min. For eggPC-LUV, flip-flop seemed to be somewhat slower compared to SUV. Halftimes for the extraction were of the same order of magnitude for SUV and LUV consisting of eggPC or POPC and were below 5 s. Again, extraction of DHE by M-CD from membranes was almost complete, as indicated by the c_m value, which was 0.97 and 0.93 for POPC and eggPC, respectively.

LUV prepared as described in Materials and Methods had a diameter of ~100 nm. Therefore, inner and outer leaflets were of the same size and one would expect a rate constant ratio k_{+1}/k_{-1} of ~1. The experimental values of 1.3 and 1.1 for eggPC- and POPC-LUV, respectively, were consistent with the expected ratio, and indicate a homogeneous distribution of DHE between both leaflets.

A striking result was obtained for DMPC: the addition of M-CD did not cause an extraction of DHE from liposomes (data not shown). Even at the highest M-CD concentration used in all experiments (24 mM) and leading to a complete extraction of DHE, e.g., from DMPC-SUV, no DHE extraction from DMPC-LUV was observed.

DISCUSSION

The purpose of this study was to establish an assay for monitoring the transbilayer movement of sterols across membranes online. For that purpose we used the extraction of DHE from membranes by cyclodextrins. β-Cyclodextrins are able to extract sterols from membranes very rapidly, and therefore provide a potential tool for measuring fast flip-flop rates. Using DHE as a reliable analog of cholesterol (see Introduction) we found that the flip-flop of this sterol in phospholipid membranes was rapid, depending on the phase state of the lipid bilayer. In the liquid-crystalline state typical for the bulk phase of biological membranes, halftimes of flip-flop were in the order of 1 min or even less.

DHE extraction assay

The assay developed in this study is based on a FRET from DHE to dansyl-PE (Wrenn et al., 1999), incorporated simultaneously into liposomal membranes. Upon addition of M-CD, DHE is extracted from vesicles, but not dansyl-PE. Extraction of DHE can be followed online by a decrease of FRET. The dansyl-PE spectrum in the absence of DHE serves as reference for a complete extraction. This offers the possibility to determine the exact amount of DHE located in the membrane upon extraction.

A major potential of the assay is to measure fast flip-flop kinetics. Its applicability depends on the quantitative relation between the rate constants for extraction of DHE by M-CD and the rate constants for transbilayer movement of DHE. Only if the extraction step is significantly faster in comparison to the transbilayer movement of DHE, the latter can be characterized by the approach. Indeed, the halftime for extraction of DHE from liposomes in the fluid phase is below 5 s, which allows for the detection of transbilayer movements with halftimes of the order of magnitude of 50 s or even faster.

Flip-flop of DHE in phospholipid membranes

The assay was applied to the extraction of DHE from SUV and LUV with different lipid compositions at 10°C. The measured kinetics was fitted to a simple model, which considered DHE flip-flop and extraction. We found that the extraction was very rapid, with halftimes well below 5 s for eggPC and POPC. The flip-flop rates at 10°C for eggPC and POPC were independent of the vesicle size, and flop halftimes were in the range of 20 to 50 s. Rates of flip-flop and extraction of DHE were significantly lower in DMPC vesicles. The flop halftime in DMPC-SUV was determined to be ~400 s at 10°C, and therefore one order of magnitude higher than that in POPC and eggPC vesicles. Nevertheless, in comparison to the transbilayer movement the extraction was still rapid, with a halftime below 30 s. These results indicate that flip-flop rates are sensitive to the phase state and/or the acyl chain length of the respective lipid. POPC vesicles are in the liquid-crystalline phase, whereas DMPC vesicles are in the gel phase at 10°C. EggPC is considered to be in an intermediate state, with a lipid dynamics resembling more the liquid-crystalline state than a gel state. The measured rate constants in SUV show that flip-flop was rapid in the liquid-crystalline phase and one order of magnitude faster than in the gel phase. The relevance of the phase state of the phospholipid membrane for dynamics of DHE is also supported by the dependence of the kinetics of M-CD-mediated DHE extraction from membrane composition. In the gel phase, the halftime of extraction was one order of magnitude higher in comparison to the liquid-crystalline state.

An interesting result of our study is that the extraction of DHE from vesicles is dependent on the vesicle curvature and on the phase state of the membrane. In contrast to DMPC-SUV, DHE could not be extracted from DMPC-LUV at 10°C. Several reasons could account for this difference in the availability of DHE for extraction. First, the high membrane curvature in SUV may perturb tight lipid packing and allows the extraction of DHE, whereas in LUV lipid ordering seems to prevent an extraction. Indeed, Loura and Pietro (1997) reported differences in steady-state anisotropy of DHE in DPPC-SUV and LUV above and below the main phase transition for varying DHE concentrations.

Anisotropy in SUV was always smaller than that in LUV for both phases, indicating different structural organizations of DHE in SUV and LUV.

Second, several authors (Harris et al., 1995; Mukherjee and Chattopadhyay, 1996; Loura and Pietro, 1997; Rukmin et al., 2001) assume the formation of transbilayer tail-to-tail dimers of sterols even at low sterol concentrations below 5 mol %. Interestingly, evidence for possible dimers is only detectable in unsonicated large vesicles but not in small sonicated vesicles, suggesting that a high membrane curvature might prevent dimer formation. The acyl chain length of phospholipids also affects formation of dimers. The formation of dimers is assumed to be due to aliphatic side chain interactions of sterols located on different leaflets. DMPC has acyl chains with 14 carbon atoms, whereas POPC has acyl chains with 16 and 18 carbon atoms. It is possible that dimer interaction is more pronounced in systems with short acyl chains than in systems with long acyl chains (Rukmin et al., 2001). Future studies have to clarify whether the existence of sterol dimers could be of consequence for the M-CD-mediated extraction of the sterols.

The fast flip-flop of DHE determined in this study is in agreement with the results of other groups. Ohvo-Rekilä et al. (2000) measured kinetics of the dequenching of 30 mol % DHE in POPC vesicles upon addition of cyclodextrins. Although it was difficult to determine DHE concentrations in the membrane from those dequenching kinetics, the data indicated a rapid flip-flop of DHE with halftimes below 40 s at 37°C. The results of a study by Backer and Dawidowicz (1981) using a cholesteroloxidase assay for eggPC-SUV containing ~40 mol % cholesterol indicate that 90% of the total cholesterol was oxidized with a halftime of ~1 min. Very recently, Leventis and Silvius (2001) measured the kinetics of β-cyclodextrin-mediated radiolabeled cholesterol transfer between LUV composed mainly of 1-stearoyl-2-oleoyl PC. Although the applied assay did not provide a sufficient time resolution, this comprehensive study on intervesicular transfer of cholesterol indicated that the flip-flop of [^{3}H]cholesterol was very rapid in those lipid membranes with a halftime of $<$1–2 min at 37°C. This estimate of halftime is in agreement with our results and supports the notion that DHE provides a faithful analog for endogenous cholesterol.

Throughout this study we assumed a homogeneous lateral distribution of DHE in both leaflets. Nevertheless, a different interpretation of the obtained results has to be considered as well. Cholesterol is known to phase-separate from phospholipids in model systems (e.g., Keller et al., 2000) and to form domains in biological membranes (see below). Thus, one may wonder whether the biphasic extraction of DHE from liposomes could originate from two different lateral domains in membranes while the transbilayer movement is faster than the M-CD extraction of DHE. Recently, Haynes et al. (2000) have found two different kinetic pools of cyclodextrin mediated extraction of cholesterol from the plasma membrane of CHO-K1 cells. However, it remained open whether these two pools correspond to lateral domains or to the exoplasmic and cytoplasmic leaflet of the plasma membrane. At the present state of our investigation, the alternative interpretation of two different lateral domains remains a possible explanation and further experiments are needed to address this issue.

Biological membranes have a more complex lipid composition than simple model systems used for most studies on sterol behavior. Although the bulk lipid phase of cell membranes is considered to be in a liquid-crystalline-like phase state, as is the case for eggPC membranes, it remains to be established whether sterol flip-flop is also very fast in cell membranes, in particular in plasma membranes of eukaryotic cells. Schroeder et al. (1991) determined a halftime of DHE flip-flop in erythrocytes of 6 min. This halftime is slower than that for DHE in eggPC liposomes as determined here. However, cellular systems contain sphingomyelin, which may slow down the transbilayer movement of sterols by lipid-lipid interactions (Ohvo-Rekilä et al., 2000). Indeed, those lipids can form or can be constituents of complexes named rafts (Simons and Ikonen, 1997). Rafts have been shown to be present in biological membranes, in particular in the plasma membrane of eukaryotic cells, and in model membranes, and exist in a liquid-ordered phase (see Simons and Ikonen, 1997; Brown, 1998; Brown and London, 1998, 2000; Dietrich et al., 2001). The flip-flop of cholesterol in those domains may be considerably slowed down with respect to that of the fluid phase. Maintaining a specific distribution of cholesterol may require specific transporters. Recent studies argue for the existence of transbilayer sterol transporters in biological membranes, as e.g., the ABC1 transporter (Lawn et al., 1999).

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